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Plecktrin Homology (PH) domains are commonly thought of as membrane-targeting modules involved in signaling pathways that bind phosphoinositides with high affinity and specificity. In a recent study of all PH domains in S. cerevisiae, only one bound PI(4,5)P2 with high affinity and specificity, while another six bound 3-phosphoinositides with moderate affinity and promiscuity; the remainder showed little or no affinity or specificity for phosphoinositides (Yu et al, 2004). The results of our human PH domain sudy thus far are comparable, with only one confirmed high affinity and PI(4,5)P2-specific and several moderate affinity and promiscuous PH domains, while the remainder are low affinity and promiscuous for phosphoinositides. Two PH domains of the moderate affinity and promiscuous class (those of FAPP1 and OSBP) possess comparable affinities for Golgi- and plama membrane-enriched phosphoinositides in vitro, although they both localize to the Golgi, not the plasma membrane in vivo. One reason for this in vivo selectivity appears to be the result of a direct interaction with the Golgi small GTPase Arf1. Moreover, the strong binding affinity of these PH domains for the monophosphoinositide PtdIns(4)P versus the diphosphoinositide PtdIns(4,5)P2 also suggests an additional phosphoinositide determinant that is currently under investigation.

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## Introduction

The primary purpose of my investigation into pleckstrin homology (PH) domains – common to a wide array of signaling proteins implicated in breast cancer – is to elucidate the manner in which PH domain recognition of phospholipids and of other proteins contributes to cellular signaling (Cesareni *et al*, 2004). Ideally, the structural determinants of these PH domain interactions and their regulation can be clarified in sufficient detail to suggest approaches for inhibiting the interactions pharmacologically. I have been pursuing several PH domain/target pairs (the latter including phosphoinositides, proteins, and an alkylphospholipid drug), employing a variety of biochemical, biophysical, and structural biological approaches to characterize the interactions in detail. Up to now, I have been largely focused on investigating the nature of these interactions quantitatively. More recently, I have been attempting to co-crystallize the PH domain and its phosphoinositide targets in order to determine three dimensional structures. By better understanding the structural basis of these protein-lipid interactions, and demonstrating a 'signaling relevance' to these interactions in breast cancer, I anticipate that these studies will succeed in identifying novel, highly-specific and well understood targets for disrupting intermolecular interactions, and contribute to structure-based drug design. Since PH domains appear to interact with both small molecules and proteins, I believe that they will offer a unique opportunity for disrupting critical protein-protein recognition events with small molecule agents that have many advantages as therapeutic agents.

Since the commonly accepted function of PH domains is to direct their 'host' protein to specific membranes in the cell, one might infer that PH domains with high, but not low, affinities for phosphoinositides should be membrane-localized *in vivo*. This generalization is clearly not universal applicable, as we have previously demonstrated that several low affinity PH domains show a surprising degree of localization to various cellular membranes in our yeast genome-wide study (Yu *et al*, 2004). Moreover, an entire class of PH domains binds multiple mono- and diphosphoinositides nonselectively (promiscuously) and with moderate affinity *in vitro,* yet are targeted to various cellular compartments *in vivo*. Members of this class include the PH domains of oxysterol-binding protein-1 (OSBP1) and four-phosphoinositide adaptor protein-1 (FAPP1), whose host proteins are involved in coordinating budding and fission events at the Golgi for the generation of cargo transporters targeted for fusion with the plasma membrane (Itoh & De Camilli, 2004; Roth 2004).

Following the format of my proposed Statement of Work, my current progress is summarized as follows:

### Task 1 : Investigate affinity and specificity of phosphoinositide binding to isolated human PH domains

I have previously described our analysis of 21 human PH domains that were selected for *in vitro* phosphoinositide binding. The majority of these PH domains (15) bind phosphoinositides promiscuously and with low binding affinity *in vitro*, and possess diffuse cytoplasmic localization *in vivo* (only the PH domains of DAGKδ and Dok1 demonstrate unexpected plasma membrane localization). By contrast, relatively few PH domains have high affinity and specificity for $PI(4,5)P_2$ or the 3-phosphoinositides $PI(3,4)P_2$ and $PI(3,4,5)P_3$. This class of PH

domains has been well documented both structurally (highly-specific interactions between amino acid side chains of PH domains and phosphates of lipid head groups) and *in vivo* (plasma membrane localization and its disruption by mutation of the PH domain's basic consensus sequence) (Ferguson *et al*, 2004; Lemmon & and Ferguson, 2000; Lemmon *et al*, 1995). Finally, the remaining class of moderate affinity and promiscuous PH domains has not yet been fully characterized structurally nor functionally. For example, the PH domains from oxysterol-binding protein-1 (OSBP1) and Four-Phosphoinositide Adaptor Protein-1 (FAPP1), have demonstrated moderate *in vitro* binding affinities, but little or no specificity, for the phosphoinositides PI(4)P and PI(4,5)$P_2$ in SPR binding studies (Levine & Munro, 1998).

I have previously reported on *in vitro* SPR binding data for the monomeric (untagged) PH domains of OSBP1 and FAPP1 for the phosphoinositides PI(4)P and PI(4,5)$P_2$. (Although multiple experiments have previously been performed using GST fusion PH domains, GST exists as a dimer in solution, which artificially enhances their relative binding affinities in SPR assays by as much as ten fold (Kavran *et al*, 1998)). These experiments have been repeated for OSBP PH (n=3) and FAPP1 PH (n=2), with consistent results in all four cases (ie.- for both phosphoinositides, the $K_d$s of OSBP PH are ~3 μM, and the $K_d$s of FAPP1 PH are 16-21 μM). OSBP PH binding to the phosphoinositide PI(3,4)$P_2$ was also measured (n=2), demonstrating a similar binding affinity (mean $K_d$ 3.4 μM) to those for PI(4)P and PI(4,5)$P_2$ (**Figure 1**). The OSBP PH domain binding data to the three phosphoinositides provide a quantitative demonstration of its moderate affinity and promiscuous binding ability. Conversely, monomeric FAPP1 PH domain binding to PI(3,4)$P_2$ was not substantially greater then binding to the PC control as measured by changes in the refractive index ($R_{max}$ 340-390 RU, n=3). Dimeric GST-FAPP1 PH demonstrated more substantial binding ($R_{max}$ 712 RU, $K_d$ 13 μM, n=1), indicating that the relative binding affinity of monomeric FAPP1 PH for PI(3,4)$P_2$ is likely to be on the order of ~65-130 μM (**data not shown**). The *in vitro* binding affinities of OSBP PH and FAPP1 PH for other mono- and diphosphoinositides will subsequently be measured.

**Task 2: Determine the subcellular localization of the PH domains**

As described in last year's report, the Alliance for Cell Signaling (AfCS) laboratory at Stanford has provided *in vivo* mouse PH domain localization data for over two-thirds of the 66 human PH domains classes listed in our original proposal (http://www.signaling-gateway.org/). The strong homology of human and mouse PH domain sequences obviates the need to repeat such a large scale *in vivo* cellular localization study with human PH domains. Rather, a handful of potentially useful PH domains selected from the AfCS study that localize to cellular membranes *in vivo* could be analyzed for their *in vitro* binding affinities at a later date.

I had previously confirmed the *in vivo* cellular localization of FAPP1 PH and OSBP PH to Golgi bodies, in agreement with previous studies (Levine & Munro, 2002; Godi *et al*, 2004). This is consistent with the host proteins' reported involvement in coordinating budding and fission events at the Golgi for the generation of cargo transporters targeted for fusion with the plasma membrane (Itoh & De Camilli, 2004; Roth 2004). Reconciling prior *in vitro* data (promiscuity in phosphoinositide binding) and *in vivo* data (Golgi localization) of OSBP PH and FAPP1 PH is an important goal in my studies (**Task 3**), which is predicted to involve a non-phosphoinositide component (Levine & Munro, 2002).

**Task 3 Putative interacting proteins of PH domains**

In my attempt to understand the role of moderate affinity and promiscuous PH domains, I will attempt to address at least two experimental observations of the OSBP/ FAPP family that remain unexplained. The first - and the subject of this task - addresses the discrepancy between *in vitro* data (promiscuity in phosphoinositide binding) and *in vivo* data (Golgi localization). More specifically, how are these PH domains correctly targeted to the Golgi *in vivo* in the absence of major differences in their *in vitro* affinity and selectivity for (Golgi-enriched) PI(4)P versus (plasma membrane-enriched and more numerous) $PI(4,5)P_2$? This question is *apropos* for all PH domains that bind phosphoinositides with low affinity and promiscuity that are nevertheless targeted to membranes and/or puncta. The second discrepancy will subsequently be discussed (**Task 4**).

Since phosphoinositide binding alone cannot account for the specific subcellular localization of these PH domains, it has been suggested that other targets, particularly protein targets, may determine their localization. For example, it was observed that Golgi targeting of the OSBP1 PH domain requires both PI(4)P and a second determinant, which (based on genetic studies) is suggested to be Arf1, a Golgi-based small GTPase (Levine & Munro, 2002). More recently, the PH domains of both OSBP1 and FAPP1 appear to directly and specifically interact with the small GTPase Arf1 *in vitro* by GST pulldown studies (Godi *et al*, 2004). I have purified myristoylated Arf1 from a DNA construct provided by Paul Randazzo's lab (Manser & Leung, 2002) and tested its potential interaction with GST-tagged FAPP1 PH and OSBP PH using GST-pulldown assays (similar to the one published in Godi *et al*, 2004). Results confirm what appears to be a relatively weak and non-robust interaction *in vitro* (**Figure 2**). Although such *in vitro* pulldown studies are plentiful in the literature, there are inherent limitations in relying solely on this approach to prove direct PH domain-protein interactions *in vivo*. My next goal is to quantitatively measure the binding affinity of this putative PH domain-Arf1 interaction.

There appears to be a consensus that a cofactor is necessary to strengthen this interaction *in vivo*, which is likely to be PI(4)P, a phosphoinositide that is highly enriched in the Golgi (Itoh & De Camilli, 2004; Godi *et al*, 2004; Levine & Munro, 2002). The challenge is to present both the myristoylated Arf1 and PI(4)P in sufficient proximity to one another to allow simultaneous interactions with the PH domains, and to maintain a cellular membrane-like structure that would convincingly demonstrate the likelihood of such an interaction occurring *in vivo*. To accomplish this task, I next plan to generate combined myrArf1/phosphoinositide (PI(4)P:PC) vesicles to quantitatively measure the binding affinity of the interaction using SPR assays. I have found one instance in the literature where myrArf1 was successfully incorporated into lipid vesicles, and I plan to broadly follow its protocol (Randazzo 1997). This project will be particularly innovative if it succeeds, as there are numerous putative PH domain-protein targets published (80 articles at last count as of mid 2005), but few have focused on potential phosphoinositide contributions, and none of these have utilized protein-lipid vesicles to measure *in vitro* binding affinities. Further crystallographic studies will advance a structural understanding of the putative complex, along with mutations that will allow me to assess the functional consequences of disrupting this mode of membrane targeting.

**Task 4: Structure Determination of selected PH domains**

One of the primary motivations of this study is to structurally characterize PH domain-ligand (protein or phosphoinositide) interactions in detail. While the discrepancy between *in vitro* binding data and *in vivo* cellular localization will be addressed, a basic question still remains unanswered concerning the similar *in vitro* binding affinities between PI(4)P and PI(4,5)$P_2$. Specifically, since phosphate groups on the inositol head group are the major PH domain determinants, how (and why) would a PH domain recognize a monophosphoinositide and diphosphoinositide with similar binding affinities? What are the structural determinants that account for PH domain promiscuity?

To address these questions, I have purified monomeric OSBP PH domain and am now attempting to crystallize it for X-ray diffraction studies, in its unliganded form, as well as complexed with the soluble, short chain (C4) derivatives of PI(4)P and PI(4,5)$P_2$. Thus far, I have observed multiple crystal hits in a variety of conditions tested in the unliganded form, and two promising crystals of OSBP PH domain liganded to PI(4)P (**Figure 3**). I am now in the process of reproducing these crystals using identical crystallization conditions, and will develop conditions to enhance crystal quality.

**PH domains as drug targets in cancer therapy**

Our original view of PH domains predicted that phosphoinositide-binding PH domains would make poor targets for pharmacological intervention, since: 1) most phosphoinositide-recognition events are essentially the same; and, 2) drugs likely to target PH domains are very highly charged, which leads to delivery problems. I have had the opportunity to test the PH domain-binding properties of perifosine, a $C_{18}$-alkylphospholipid drug that has recently completed phase I trials (Van Ummersen *et al*, 2004; Crul *et al*, 2004) as an anti-cancer agent. Earlier immunoprecipitation studies suggested that perifosine specifically inhibits Ser/Thr phosphorylation and kinase activation of Akt1/PKB *in vivo* and *in vitro* (Kondapaka *et al*, 2003). Myristoylated Akt1/PKB, which is targeted directly to the plasma membrane in a PH domain-independent manner, is unaffected by perifosine treatment. I therefore surmised that perifosine might act by directly interfering with phosphoinositide binding of the PKB PH domain. In my last report, I presented SPR binding data suggesting that perifosine specifically competes with phosphoinositides for binding to the PH domain of Akt1/PKB ($EC_{50}$ 26 μM), while it competes substantially less for binding to the PH domain of PLCδ, and not at all for the PH domains of DAPP1 and FAPP1. These studies indicate that perifosine likely binds directly to the phosphoinositide-binding site of the PKB PH domain. Follow up studies to determine the binding affinity of the Akt1/PKB PH-perifosine interaction using isothermal titration calorimetry (ITC) were difficult to perform due to the relatively high concentrations of PH domain required for the study, but confirmed the relatively low binding affinity (~30 μM) of the interaction (**Figure 4**). Thus, although perifosine interacts specifically with PKB PH *in vitro*, other means appear to be necessary – perhaps other segments of Akt1/PKB or even an additional cofactor – to "boost" the binding affinity of this interaction.

## Key Research Accomplishments

- The binding affinities of monomeric OSBP PH and FAPP PH for PI(4)P and PI(4,5)$P_2$ using SPR assays was repeated, confirming previous results.
- The binding affinity of OSBP PH for PI(3,4)$P_2$ was measured, demonstrating the promiscuity of OSBP PH for phosphoinositides quantitatively *in vitro*.
- The binding affinities of dimeric GST-FAPP1 PH and monomeric FAPP1 PH for PI(3,4)$P_2$ were measured, indicative of their substantially weaker binding vis à vis PI(4)P and PI(4,5)$P_2$.
- Weak myrArf1 interactions with OSBP PH and FAPP1 PH were demonstrated *in vitro* using GST pulldown assays .
- Multiple putative hits of crystallized OSBP PH domain alone, and OSBP PH co-crystallized with short chain C4-PI(4)P and C4-PI(4,5)$P_2$, were discovered.
- The alkylphospholipid drug perifosine has a relatively low binding affinity for PKB PH according to ITC studies, despite its rather strong selectivity for the domain.

## Reportable Outcomes

- Completed early draft of 'PH domain-protein interactions' review article.
- Cloned OSBP PH-2TK and FAPP1 PH-2TK constructs for dot blots experiments.
- Developed Western blot for myristoylated Arf1 detection.
- Developing putative protein crystals.
- Completed *in vitro* binding analysis of Akt1/PKB PH-perifosine interaction.

## Conclusion

Consistent with our previous yeast genome wide study (Yu *et al*, 2004), the vast majority of PH domains demonstrate low affinity, promiscuous binding of phosphoinositides, and relatively few demonstrate high affinity, phosphoinositide-specific binding. A third relatively understudied group of PH domains possess moderate affinity and promiscuity for phosphoinositides, and often target to particular cellular compartments *in vivo*. One group from this class, OSBP/FAPP family, target to the Golgi *in vivo*, and are related to the yeast PH domains of Osh proteins (Lehto *et al*, 2001). Interestingly, at least two members of this group (FAPP2 and OSBP2) have been associated with breast cancer development or progression (Fournier *et al*, 1999; Scanlan *et al*, 2001).

I have continued my *in vitro* analysis of monomeric OSBP PH and FAPP PH by: (1) confirming that their relative binding affinities for Golgi-specific PI(4)P are very similar to that of plasma membrane-specific PI(4,5)$P_2$; (2) demonstrating that the binding affinity of monomeric OSBP PH for PI(3,4)$P_2$ is comparable to that for PI(4)P and PI(4,5)$P_2$ (indicative of its intrinsic promiscuity for phosphoinositides), whereas the binding affinity of monomeric FAPP1 PH for PI(3,4)$P_2$ is much weaker. The *in vitro* binding affinities of OSBP PH and FAPP1 PH for other mono- and diphosphoinositides will subsequently be measured.

It has been suggested that a non-phosphoinositide cofactor is required to explain the discrepancy that members of the OSBP/FAPP family are targeted to the Golgi *in vivo* in the

absence of any difference in the *in vitro* binding affinities for (Golgi-enriched) PI(4)P versus (PM-enriched) PI(4,5)$P_2$ (Godi *et al*, 2004). The Golgi-based GTPase Arf1 has been proposed to be the cofactor based on *in vitro* pulldown studies, results that are consistent with my study. To quantify this interaction, I will purify myristoylated Arf1 and combine it with PI(4)P (in a PC background) to create protein/lipid vesicles. These vesicles should be amenable to binding affinity determination by SPR assays.

A second unresolved question involving the OSBP/FAPP PH family concerns the identification of structural determinants that account for PH domain promiscuity given that: 1) the relative binding affinities for the PH domains for the monophosphoinositide PI(4)P and the diphosphoinositide PI(4,5)$P_2$ are similar; and 2) phosphate groups on the inositol head group are major PH domain determinants. I am attempting to solve the crystal structure of OSBP PH domain alone and complexed with the phosphoinositides PI(4)P and PI(4,5)$P_2$ to address this issue. I have observed multiple crystals in a variety of conditions tested in the unliganded form, and two promising crystals of OSBP PH domain liganded to PI(4)P, which are currently under further development.

Finally, after having demonstrated the specificity of the anticancer drug perifosine for Akt1/PKB PH for the last reporting period, I have currently demonstrated perifosine's low binding affinity for Akt1/PKB PH as measured by ITC. Understanding the structural basis for this PH domain-specific interaction will allow me to elucidate the determinants involved, contributing to the quest for an effective anticancer therapy.

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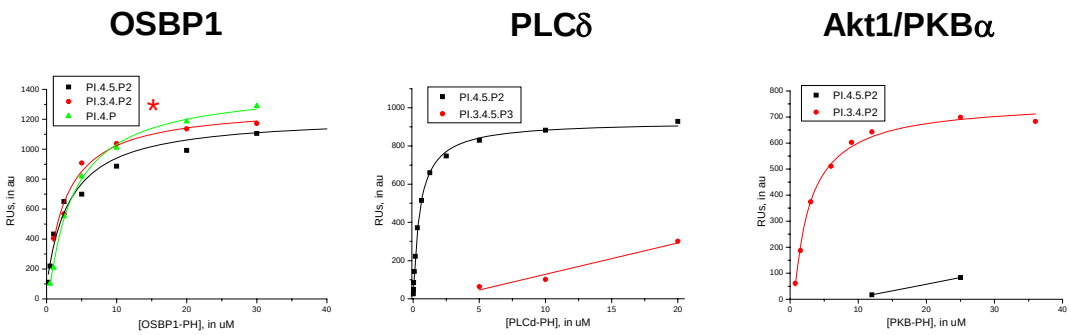


**Figure 1**
**SPR binding data for OSBP PH suggest promiscuity for phosphoinositides.**
SPR binding assay protocol detailed in Yu *et al*, 2004.
BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of Y=(Rmax*((1/Kd)*X)/(1+((1/Kd)*X)))+cf, where Rmax is the maximal response and cf is the correction factor.

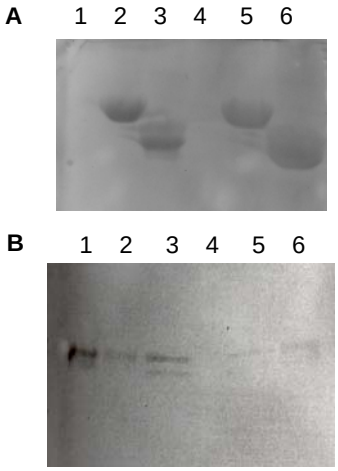


**Figure 2**
**GST pulldown data suggest weak myrArf1 interactions with OSBP and FAPP1 PH**.
Recombinant myristoylated Arf1 was expressed and purified as previously described (Cesareni *et al*, 2004). GST-OSBP-PH and GST-FAPP1-PH were expressed and purified as previous GST-tagged proteins, with the exception that they were retained immobilized, and not eluted, on the glutathione-Sepharose beads (Lemmon *et al*, 1995; Yu *et al*, 2004). myrArf1 was loaded with 100 μM GTP-γS (the non-hydrolyzable analog of GTP) or GDP by a 1 hr incubation at $32^0$C in HEPES loading buffer, followed by a 1 hr. incubation with GST-PH domain immobilized on glutathione-Sepharose beads at RT, as described previously (Godi *et al*, 2004). Beads were collected by low-speed centrifugation, washes 3X, and resuspended in 3X sample buffer. Sample was boiled for 5 min at $95^0$C, and run on a 15% SDS-PAGE. Proteins were transferred to nitrocellulose paper by Western blot, blocked for 10 min in Blotto buffer with 5% dry milk, and incubated with **A)** mouse anti-GST antibody (1:1000), or **B)** goat anti-Arf1 antibody (1:1000) in 1X PBS O/N at $4^0$C. The blot was washed 3X with 1X PBS, followed by a 1 hr incubation at $4^0$C with secondary antibody (rabbit anti-mouse and donkey anti-goat, respectively), and washed again 3X. Finally, the blot was developed with ECL reagents, as per the manufacturer's instructions.
Lanes 1-6: myrArf1 alone, GTP-loaded myrArf1 + GST-OSBP-PH, GTP-loaded myrArf1 + GST-FAPP1-PH, prestained Mol. Wt. marker, GDP-loaded myrArf1 + GST-OSBP-PH, GDP-loaded myrArf1 + GST-FAPP1-PH

**Figure 3**
**Putative OSBP PH Crystals**
Untagged OSBP PH domain was cloned into pET 11a and purified using similar procedures as prior untagged PH domains (Lemmon *et al*, 1995). Protein was concentrated and buffer exchanged into 10 mM MES pH 6.0. Protein was prepared for crystallography either uncomplexed, or complexed with short chain (C4) PtdIns(4)P or PtdIns(4,5)$P_2$ in a 1:1.5 ratio. Protein was pipetted onto siliconized glass slides (1 μL protein + 1 μL mother liquor) using the hanging-drop method with various laboratory-made and manufacturer (Hamptons and Emerald Biosciences, Inc.) crystal screens.
Above are examples of some promising leads; the top three are OSBP PH complexed with PtdIns(4)P, while the remainder are uncomplexed OSBP PH.

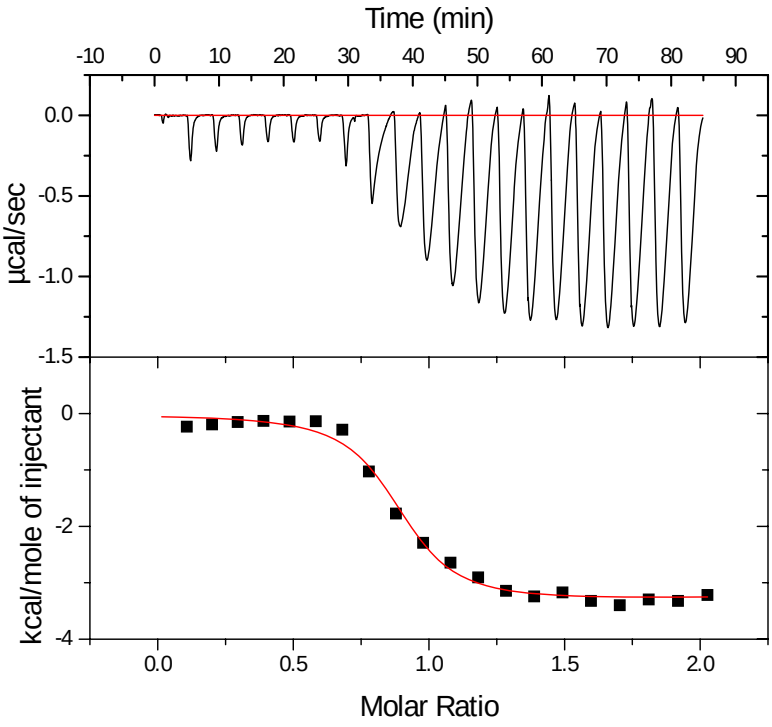


**Figure 4**
**ITC binding data suggest perifosine's weak binding affinity for Akt1/PKBα PH**.
GST-Akt1/PKBα PH was expressed and purified as previously described (Thomas *et al*, 2002). The protein was dialyzed O/N at $4^0$C into 20 mM HEPES pH 7.5, 100mM NaCl, 1 mM DTT and concentrated to 360 µM. The protein was added to the 2 mL ITC cell, while perifosine (3.6 mM) filled the 250 µL syringe. Each injection was 12.5 µL for a total of 20 full injections. The binding curve was fitted with the two-sets of sites model in Origin.